SYNOPSIS OF THESIS

OLFACTORY CONTROL OF *DROSOPHILA* BLOOD-PROGENITOR HOMEOSTASIS AND DEVELOPMENT VIA REDOX REGULATION

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SYNOPSIS

Chapter 1: Introduction

Drosophila melanogaster has been used as a developmental model for decades. As our understanding of the complexities of development expands, the significant degree of conservation between *Drosophila* and vertebrate systems has become more apparent (1). During *Drosophila* larval development, lymph gland harbours the blood-progenitor cells akin to the mammalian common-myeloid progenitors (CMP). It disintegrates during the pupal stage and contributes to sessile and circulatory blood cell pool of adult animal. Various signaling molecules and metabolites produced by the local niche (posterior signaling centre, PSC) and differentiating hemocytes, and systemic cues originating from the brain and fat body affect progenitor maintenance (2). Similar to CMPs, the blood-progenitor cells of lymph gland maintain elevated levels of reactive oxygen species (ROS) whose homeostasis is necessary for their development (3–5). While physiological ROS sensitizes progenitor-cells to differentiation cues, its excessive production causes oxidative stress and loss of progenitor homeostasis (3,6). Therefore, the blood-progenitors possess robust mechanisms to maintain their ROS status, but our understanding in this regard remains limited. ROS levels in the hematopoietic stem and progenitor cells are maintained by controlling ROS production and scavenging. A key source of ROS generation in cells is tricarboxylic acid (TCA) cycle and OXPHOS (7–9). The second mode of ROS regulation, ROS scavenging is accomplished by the antioxidant enzymes and compounds like catalase, superoxide dismutase and glutathione etc (10). The goal of this thesis is to characterize the mechanisms and importance of ROS regulation during blood-progenitor development. This work demonstrates the utilization of the sensory cues of olfaction and downstream neuronal release of GABA and its catabolism in controlling blood-progenitor development and lymph gland growth via regulating ROS homeostasis. Olfaction derived GABA catabolism acts as a central regulator of blood-progenitor development during homeostasis (11) and immune-response (11,12) conditions.

The current study titled **'Olfactory control of blood-progenitor homeostasis and development via ROS regulation'** is divided into seven chapters.

The first chapter highlights and reviews the literature on blood-progenitor development and ROS regulation, as well as discusses open questions. The second chapter describes the objectives of the study highlighting the role of olfaction and downstream systemic GABA catabolism in blood-progenitor development by governing lymph gland ROS homeostasis. Chapter three to five discusses the findings of the thesis. Chapter three of the thesis discussing about the role of olfaction derived GABA catabolism in regulating blood progenitor ROS homeostasis and lymph gland growth is published in journal Development (11). Chapter four highlights the mechanism of glutathione (GSH) biosynthesis by olfaction derived GABA catabolism. Chapter five deals with standardizing and establishing the metabolic flux analysis protocols in *Drosophila* blood-progenitor cells and lymph glands. The sixth chapter deals with material and methods of the thesis, *Drosophila* genetics, immunohistochemistry, confocal imaging and mass-spectrometry (LC/MS) based metabolite analysis has been predominantly utilized to achieve the objectives of the thesis. The work described in this thesis elucidates the role of environment odor sensing and its impact on metabolic pathway regulation and ROS homeostasis during *Drosophila* blood-progenitor development. This work for the first time has utilized the LCMS based flux analysis approach in understanding metabolic status of *Drosophila* lymph gland progenitor cells. The seventh chapter highlights future directions and our contribution to current understanding of similar mechanisms during blood-progenitor development in both invertebrates and vertebrates.

Motivation for this thesis

It is widely known that metabolism is highly dynamic and any small perturbations in the cells lead to metabolic alterations. The hematopoietic niche of the vertebrate system is significantly more complex (13,14) and also relatively inaccessible. Thus, gaining *in vivo* access to metabolic states of blood compartment during its development and understanding the interactions with signaling modules is difficult. While work to address these problems are ongoing, simpler model systems with conserved haematological functions have emerged as significant tools for advancing the idea of metabolism in the control of blood progenitor development. Common myeloid progenitors (CMPs) in mammals maintain increased levels of ROS, which play

important role in stem cell state and function (5,15). Interestingly, blood-progenitors in *Drosophila* also maintain elevated levels of ROS, which primes the progenitor towards differentiation cues. Any reduction in ROS levels during blood-progenitor development leads to loss of progenitor differentiation while, any ectopic production of ROS leads to loss of progenitor homeostasis (3). Any understanding of metabolic or signaling events that enable the sustenance of this fine redox balance and blood-progenitor development remains elusive. The ability to recognize and characterize the roles of the molecular machinery that supports developmental processes is substantially facilitated by the genetic resources available in *Drosophila*. The GAL-4/UAS system is utilized to manage the over-expression or silencing of genes using RNA interference in a spatially and temporally controlled manner. Overall, *Drosophila* lymph gland provides a highly relevant developmental model for investigating the mechanisms that control blood-progenitor maintenance and differentiation. Therefore, mechanisms underlying blood progenitors development coupled to redox homeostasis forms the central focus of current thesis.

Chapter 2: Objectives

This thesis work describes **"the odor mediated control of blood-progenitor development and maintenance via ROS regulation".** *Drosophila* blood-progenitors are an integral component of the hematopoietic organ, lymph gland and various signaling and metabolic cues regulate the development of this compartment. Previous studies have implicated olfaction and its downstream signaling mediated release of neuronally-derived GABA in progenitor maintenance (16) and immune response (12). We have found that olfaction derived GABA release and its catabolism in blood progenitors control lymph gland growth and blood-progenitor differentiation by governing lymph gland ROS homeostasis. Animals that fail to smell also fail to sustain ROS homeostasis, which leads to lymph gland growth retardation.

The specific objectives of this thesis are described below:

1. Investigating the role of olfaction derived GABA catabolism in regulating blood progenitor development and lymph gland growth via controlling ROS homeostasis

2. Exploring and delineating the mechanism of involvement of olfaction derived GABA catabolism in regulation of glutathione (GSH) biosynthesis
3. Standardizing the protocol of metabolic flux analysis in *Drosophila* blood-progenitor cells by utilizing Liquid Chromatography/Mass-Spectrometry (LC/MS) based approach.

Chapter 3-5: Results

3. **Investigating the role of olfaction derived GABA catabolism in regulating blood progenitor development and lymph gland growth via controlling ROS homeostasis**. Previous studies (12,16) have shown the importance of odor-sensing and downstream systemic GABA release in blood-progenitor development in homeostatic and immune-response conditions. We found that odor-sensing is important to maintain blood-progenitor ROS homeostasis. Animals that fail to smell (*Orco-Gal4*, *UAS-Hid*), fail to sustain TCA activity and ROS levels in the blood-progenitor cells, and consequently retarded lymph gland growth. Perturbation of GABA catabolic pathway (*UAS-Gat*^{RNAi} and *UAS-Ssadh*^{RNAi}) in the blood-progenitor cell by utilizing progenitor specific driver (*domeMeso-Gal4;UAS-GFP*) leads to smaller lymph gland size accompanied by an elevation of ROS levels. We investigated the mechanism of ROS generation regulation in the blood-progenitor cells. Since, TCA is a major contribution of ROS generation in a cell, TCA activity was analysed in GABA catabolism perturbed backgrounds. Our data show that loss of GABA catabolic pathway (*domeMeso-Gal4;UAS-GFP*, *UAS-Gat*^{RNAi} and *UAS-Ssadh*^{RNAi}) in the progenitor cells leads to reduction in active pyruvate dehydrogenase kinase (pPDK) and inactive pyruvate dehydrogenase (pPDH), while the levels of total PDK and total PDH does not show any change. PDH is the rate-limiting enzyme that connects pyruvate to the TCA cycle and to oxidative phosphorylation. Thus, GABA catabolism via PDK activation maintains TCA activity and blood progenitor ROS homeostasis, and supports normal lymph gland growth. Consequently, any perturbations in GABA catabolism lead to heightened TCA activity and impaired ROS homeostasis, which leads to lymph gland growth retardation. Our work shows TCA cycle activity and ROS as one of the prime regulator of lymph gland growth. Further, GABA catabolism mediated restriction on TCA cycle activity is important for progenitor homeostasis and lamellocyte formation upon parasitic wasp-infections (Fig. 1) (11).

4. **Exploring and delineating the mechanism of involvement of olfaction derived GABA catabolism in regulation of glutathione (GSH) biosynthesis.** This part of the thesis uncovers the function of olfaction derived GABA catabolism in regulating blood-progenitor GSH synthesis by shuttling pyruvate metabolism. We investigated the mechanisms for ROS scavenging regulation by GABA catabolism and discovered that loss of GABA transporter (GAT) and GABA catabolising enzyme *Ssadh*^{RNAi} resulted in a decrease in GSH levels in lymph glands, an important antioxidant responsible for scavenging reactive oxygen species (ROS). Glutathione is a tripeptide, synthesized from glutamate, cysteine and glycine (17). We found that in the blood-progenitor cells GABA catabolism modulate cysteine levels, which further regulates GSH biosynthesis (Fig. 1). *DomeMeso-GFP>Gat*^{RNAi} and *domeMeso-GFP>Ssadh*^{RNAi} lead to reduction in blood-progenitor cysteine levels, but did not show any change in glutamate and glycine levels, detected by antibodies against glutamate and cysteine. Thus, Cysteine is a key component in GSH production, and its availability is controlled by GABA catabolism. Serine, along with methionine, is known to contribute to cysteine production in cells via the transsulfuration pathway (18). We independently checked the involvement of both serine and methionine in GSH synthesis regulation by GABA catabolism. Our results demonstrated that metabolically, GABA catabolism derived succinate control serine levels in the progenitor cells, which contribute to cysteine and downstream GSH synthesis and thus maintain ROS homeostasis of the blood-progenitor compartment (Fig. 1).

5. **Standardizing the protocol of metabolic flux analysis in *Drosophila* blood-progenitor cells by utilizing Liquid Chromatography/Mass-Spectrometry (LC/MS) approach**. This part of the work deals with utilizing the mass-spectrometry based approach to establish ^{13}C-isotope labelling and metabolic flux analysis in the lymph gland blood-progenitor cells. While recent evidences suggests the distinct metabolic requirements of myeloid cells across systems ranging from mammals to invertebrates, a comprehensive study of this scale has not been attempted (19,20). The current thesis work describes the utilization of LC/MS/MS based methods and ^{13}C-isotope labelling for analysing TCA cycle metabolites, amino acids and GSH:GSSG in lymph gland progenitor cells. Lymph gland are dissected and incubated with 10mM U^{13}C-glucose or 10mM U^{13}C-pyruvate isotopic label in PBS for specific time points

and the samples are processed for metabolite extraction. The method of (21,22) are utilized for TCA metabolite analysis.

We show that olfaction derived GABA release and its downstream catabolism in blood-progenitor cells regulate lymph gland growth and blood-progenitor development by regulating ROS homeostasis. A comprehensive study of metabolic pathways and metabolites during blood progenitor development is achieved by utilizing genetics, immunohistochemistry, microscopy (imaging) and mass-spectrometry based approaches. Our findings describe that olfaction-derived GABA catabolism modulates pyruvate metabolism in blood-progenitor cells to maintain ROS homeostasis and blood-progenitor development.

Chapter 6: Materials and Methods

To achieve the above mentioned objectives following materials and methods are utilized, which are briefly outlined here. *Drosophila melanogaster Gal4* and RNA*i* stocks are either procured from BDSC, VDRC or requested from various research laboratories. All fly stocks are reared on corn meal agar food medium with yeast supplementation at 25°C incubator unless specified. The crosses involving RNA*i* lines are maintained at 29ºC to maximize the efficacy of the *Gal4/UAS RNAi* system. *Leptopilina boulardi* (parasitic wasps) are cultured and maintained on w^{1118}. For ROS detection lymph gland were stained with DHE (Sigma, 1:1000 in PBS). For Immunohistochemistry, lymph glands isolated from larvae are stained. Immunostaining on lymph gland is performed majorly with the following primary antibodies: rabbit αpPDHK1(1:200, #11597 SAB), rabbit αpPDH (1:250, abcam #177461), mouse-αPDH (Abcam, ab110334, 1:250), mouse-αPDK (Abcam, ab110025, 1:500), mouse αCysteine (1:20 # sc-69954), Mouse α Myospheroid (1:100 # CF.6G11), rabbit αGABA (1:100, sigma #A2052), rabbit αGlutathione (1:100, abcam #5543), mouse-αGlutamate (1:100, #ab9440). The following secondary antibodies are used at 1:500 dilutions: FITC, Cy3 and Cy5 (Jackson Immuno Research Laboratories and Invitrogen). Samples are mounted with Vectashield (Vector Laboratories). Immuno-stained lymph gland images are acquired using Olympus FV3000 Confocal Microscopy with 40X oil-immersion objective. All images are quantified using ImageJ software. Roughly, middle two confocal Z-stacks are merged and lymph gland area is marked and measured. This is done for respective zones and the area is represented in percent values. Controls are

analysed in parallel to the sample conditions every time. For quantifying mean intensities in lymph gland, the relevant stacks of the lymph gland images are selected, the area to be measured per lobe is defined using the select tool and is then processed for intensity measurements by considering mean intensity values. For all intensity quantifications, the laser setting for each individual experimental set-up is kept constant. For Metabolite supplementation experiments, Succinate (Sodium succinate dibasic hexahydrate, Sigma #SLBM6312V), Serine (0.1%, Sigma), Methionine (0.1%, Sigma), N-Acetylcysteine (0.1%, Sigma) and GABA (3%, Sigma) enriched diets are prepared by supplementing regular fly food with respective amounts by weight/volume measures of succinate to achieve 3% concentrations. Eggs are collected in these supplemented diets and reared until analysis of their respective tissues. For Mass-spectrometry based LCMS analysis, five lymph glands per sample are dissected from the larval stage of interest. Metabolites are isolated from lymph glands using specific methods, and the samples are then subjected to LC/MS analysis. The acquired data is processed further by either MassHunter or MultiQuant software. Statistical analyses are performed using GraphPad Prism software and Microsoft Excel.

Chapter 7: Discussion

It has been well established that ROS as a signaling entity are critical for blood-progenitor development and maintenance (10,23,24). However, mechanisms limiting ROS levels that are critical for its functioning in myeloid progenitor cells remain mostly unclear. We find that blood development in *Drosophila* is strikingly influenced by sensory cues of olfactory in origin. With the current work, we present the developmental role for olfaction in blood progenitor redox balance. Recent research has demonstrated the importance of GABA in myeloid immunity, specifically in metabolic programming of myeloid cells during innate immune training (25–28). These findings point to similarities between the myeloid systems of mammals and *Drosophila*. Our findings in the hematopoietic system of Drosophila emphasize many developmental roles of GABA in myeloid development, progenitor homeostasis, and immunity. Upon olfactory stimulation, neuronally derived GABA and its metabolism is linked to limiting pyruvate's entry into the TCA cycle and this restricts TCA derived precocious ROS production (11) and leads to generation of antioxidant, glutathione. Our findings show that myeloid metabolism and ROS balance by controlling both the ROS generation and

scavenging axis are sensitive to olfaction-derived GABA. This axis is required for appropriate lymph gland growth and development.

These findings not only reveal novel activities of neurotransmitters like GABA in hematopoiesis, but also expand our understanding of the relationship between animal odor-sensing and systemic modulation of blood progenitor metabolism and redox balance.

Significance and future prospects of the present research

Drosophila and the mammalian hematopoietic system share many conserved characteristics. As a result, the findings in *Drosophila* blood development are intriguing prospects with a lot of potential for translation into the mammalian hematopoietic system as well (19,29). While the *Drosophila* hematopoietic system provides insight into myeloid-like blood progenitor development and immunity, we cannot expect it to entirely mirror the vertebrate immune system. Mammalian hematopoietic niche, bone marrow, is more complex and is controlled by a number of additional modulators. As a result, the metabolic pathways and cross-talks found in this work may have additional layers of complexity during blood-development in higher organisms and the identified mechanism in the fly model must be independently confirmed in vertebrate model systems like mice. Moreover, the complexity of the vertebrate immune system due to presence of lymphoid arm and the existence of additional blood cell types, makes it difficult to delineate mechanisms specific to the myeloid or lymphoid arm. The first step could be to test the existence of these processes or pathways in mammalian systems. Nonetheless, *Drosophila* model provides a multitude of genetic tools for investigating mechanisms underlying development and disease. As we continue to learn more about blood formation and reveal fundamental mechanisms in simpler systems like *Drosophila*, this research can be utilized to develop techniques and approaches for dealing with complex vertebrate systems.

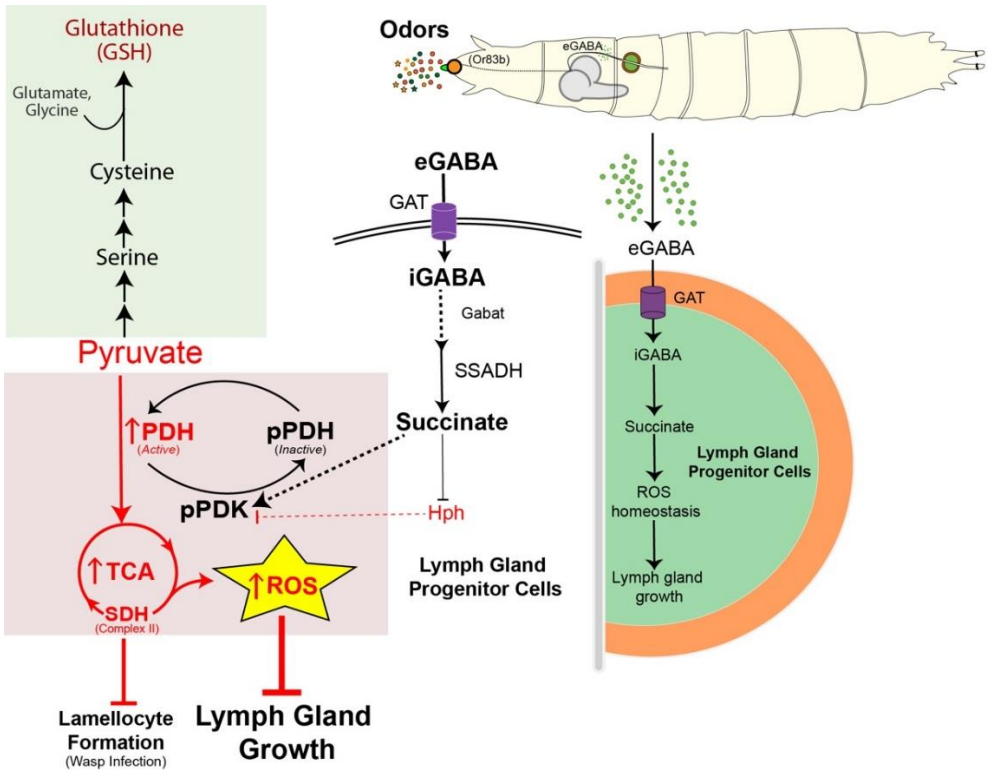

Figure 1. **Olfaction-derived systemic GABA and its catabolism in lymph gland ROS homeostasis and growth control.** The model describes the importance of olfaction-derived GABA metabolism in lymph gland growth control. All elements that repress growth are shown in red, while positive regulators of growth are shown in black. Blood progenitor cells of the *Drosophila* larval lymph gland maintain ROS in them that is derived from the TCA cycle. However, heightened or uncontrolled TCA cycle activity, leading to increased ROS production in progenitor cells, abrogates lymph gland growth and development. GABA catabolism derived TCA regulation is also important for lamellocyte formation during immune response conditions. Secondly, in the blood-progenitor cells, GABA catabolism derived succinate shuttles pyruvate towards gluconeogenic arm and leads to serine and consequently glutathione (GSH) formation.

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